Electron Microscopic Studies of *Mycoplasma pulmonis* (Negroni Strain)

By T. TANAKA* AND D. A. WOODS

*Department of Biological Ultrastructure, The Imperial Cancer Research Fund,
Lincoln's Inn Fields, London W.C. 2*

(Accepted for publication 4 August 1970)

SUMMARY

Human foetal kidney cell cultures and agar and broth media were inoculated with *Mycoplasma pulmonis* (Negroni), a rodent pathogen, and were examined by electron microscopy.

In the tissue cell cultures, abundant extracellular mature mycoplasmas and elementary bodies were seen. The average diameter of elementary bodies was 99 nm. The average long and short axes of mycoplasmas growing on agar measured 800 and 600 nm. respectively.

Whole mounted broth-cultured mycoplasmas consisted of three principal cell types: large pleomorphic cells (up to 1500 nm. in diameter), filamentous forms, and elementary bodies (less than 180 nm. in diameter). Production of elementary bodies by budding from the mature cells was the normal method of multiplication. The life cycle was estimated to be 46 ± 7 h. under optimum conditions. Immunoelectron microscopy demonstrated positive reactions only with specific antisera.

INTRODUCTION

Negroni (1964) reported the isolation of a filterable cytopathic agent from human leukaemic bone marrow. This agent was later proved to be a strain of *Mycoplasma pulmonis*, a rodent pathogen (Grist & Fallon, 1964; Fallon, *et al.* 1965; Butler & Leach, 1966; Sabin, 1967; Somerson, Reich, Chanock & Weissman, 1967). The significance of the relationship between mycoplasma and human leukaemia remains a subject for conjecture (Beale, Christophinis & Negroni, 1968; Fallon & Jackson, 1968).

This report describes the electron microscopic characteristics of the Negroni agent. Fallon & Jackson (1967) noticed serological differences among several strains of *Mycoplasma pulmonis* and emphasized that 'not all strains of *M. pulmonis* are identical, and subtypes of this species exist'. They used ferritin-conjugated anti-Negroni agent antisera, prepared in two separate laboratories, against a conventional strain of *M. pulmonis* and also strain 880, reported to be a strain of *M. pulmonis* (Butler & Leach, 1966; Sabin, 1967; Somerson *et al.* 1967) and a causative agent of human leukaemia (Grace, Horoszewicz, Stim, Mirand & James, 1965).

Mycoplasmas have been considerably confused with certain viruses in the interpretation of electron micrographs, as can be seen in the reports of Negroni (1964) and Inman, Woods & Negroni (1964). This is because of the production by mycoplasmas

* Present address: Department of Experimental Pathology and Cancer Research, School of Medicine, University of Leeds, Leeds 2.

of elementary bodies which closely resemble some virus particles. Greater knowledge of the production of elementary bodies is therefore urgently required, but little work has been done on this. Morowitz & Maniloff (1966) and Furness (1968) have studied the growth cycle of *Mycoplasma gallisepticum* A 5969 and *M. orale* respectively. However, these two species of mycoplasma divide mainly by binary fission, and only one available report, on the development of *M. gallisepticum* s6 (Dutta, Dierks & Pomeroy, 1965), describes reproductive activity similar to that seen in *M. pulmonis* (Negroni).

This paper describes some aspects of the morphology, surface antigenicity and life cycle of the Negroni agent, particularly the mode of production of elementary bodies, as revealed by electron microscopy.

METHODS

Material from tissue culture and agar medium

Mycoplasma, maintained in broth medium, were inoculated into human foetal kidney cultures. The broth medium used consisted of 70 % (v/v) broth, 20 % (v/v) unheated horse serum, 10 % (w/v) yeast extract, 1 % (w/v) thallium acetate and penicillin (0.1 ml. containing 10,000 units to each ml. medium). Forty-eight h. later any cells remaining on the culture-tube walls were dislodged and the cell suspension was sedimented at 800 *g*. The sediment was fixed in Millonig's buffered phosphate (pH 7.2) containing 2 % (w/v) osmium tetroxide (Millonig, 1962), dehydrated in ethanol, embedded in Maraglas and sectioned. Mycoplasma colonies growing on agar were fixed *in situ*, and thin strips from the surface of the gel were embedded for sectioning. Sections were stained with a 25 % (w/v) solution of uranyl acetate in methanol, followed by Reynolds lead citrate (Reynolds, 1963). The long and short axes of 229 sectioned mature mycoplasma cells in the agar colonies were measured to find the mean cell size.

Material from broth medium

Broth cultures of Negroni strain containing 10^6 colony-forming units/ml. were incubated at 37°; the number of colony-forming units remained constant until about the fifth day of culture, when it began to decline.

The broth cultures were centrifuged at 26,000 rev./min. in the Spinco rotor 30 for 1 h. at 4°. A 2 % (w/v) aqueous solution of phosphotungstic acid, adjusted to pH 7.1 with potassium hydroxide, was used for negative contrast preparations. In order to spread the phosphotungstate evenly on the grids, approximately 0.05 % (w/v) bovine serum albumin was added to the solution immediately before use. Droplets of the sedimented pellet mixed with phosphotungstate solution were applied to grids bearing carbon support films. The cultures were examined by electron microscopy every day for 10 days. Some specimens were examined without contrast medium.

Some specimens of the mycoplasma were fixed prior to their preparation for negative contrast by a method similar to that employed by De Harven & Friend (1964). Ultracentrifuged pellets from broth culture were resuspended in 3 ml. of 0.12 M-sodium citrate (pH 7.0) which was isotonic with the mycoplasma culture medium. The suspension was squirted into an equal volume of 0.5 % (w/v) osmium tetroxide in Millonig's phosphate buffer (pH 7.2) and resedimented at 25,000 rev./min. in the Spinco rotor 50.

To study the life cycle of Negroni agent, broth cultures containing 10^6 colony-

forming units/ml. were filtered with a 450 nm. Millipore filter, and then with a 220 nm. filter. The resulting filtrate free of mature mycoplasma was divided into samples for hourly observations by electron microscopy. These filtrates were incubated at 37°. The optimum pH for culture was found to be pH 7·8.

For immunoferritin experiments, a sandwich technique was employed which required only one conjugated antibody. Goat antirabbit globulin was conjugated with ferritin according to the method of Singer & Schick (1961). The test organisms used were *Mycoplasma pneumoniae* (Eaton agent) and the following strains of *M. pulmonis* M1; Cheng; 880; Negroni. Rabbit immune serum globulins were prepared against the above mycoplasmas in the Imperial Cancer Research Fund, except for those against Negroni and 880 strains which were prepared at two different institutes, Imperial Cancer Research Fund, London (L) and Ruchill Hospital, Glasgow (G).

1. Rabbit anti-Negroni	(L)
2. Rabbit pre-immunization	(L)
3. Rabbit anti-Negroni	(G)
4. Rabbit pre-immunization	(G)
5. Rabbit anti-880	(G)

Broth cultures of mycoplasma were pelleted and fixed in formal calcium acetate (Douglas, Gottlieb, Strauss & Spicer, 1966). Each of the mycoplasmas listed above was allowed to react with each serum, followed by the conjugate. Controls were set up as follows:

1. Specific sera were followed by unconjugated ferritin alone.
2. Specific serum was omitted and ferritin conjugated antibody was used alone.
3. Unconjugated goat serum was used before the ferritin conjugate, as a blockade.
4. Specific serum was replaced by a saline wash.

Agar and broth cultures of the Negroni strain and human foetal kidney cultures were supplied by Dr G. Negroni, Imperial Cancer Research Fund, London. Rabbit immune sera against mycoplasmas were supplied by Dr Negroni and Dr R. J. Fallon, Ruchill Hospital, Glasgow.

RESULTS

Cultures

Abundant extracellular mature mycoplasmas were sedimented with the culture cells (Pl. 1, fig. 1). The plasma membrane was approximately 7·5 nm. thick and had a triple-layered unit-membrane structure. It was not possible to decide whether the protein lamellae of the membrane were of equal or unequal thickness. Numerous elementary bodies of average diameter 99 nm. were found scattered among the mature mycoplasmas. They had many of the morphological characteristics of some viruses, namely uniformity of size and shape, a dense core and an outer membrane. The smallest particles among them measured 53 nm. (Pl. 1, fig. 2).

Cells in sectioned, agar-cultivated colonies grew densely in agar and appeared globular in shape. The long and short axes of the organisms had mean lengths of 800 and 600 nm. respectively.

Examination of whole organisms

Three principal cell types occurred during the reproductive cycle in broth medium: (a) large pleomorphic cells, (b) filamentous forms and (c) elementary bodies.

Whole organisms, mounted on support films with or without contrast medium, appeared pleomorphic, the largest measuring 1500 nm. in diameter. The organisms exhibited a relatively stout membrane structure, 12.5 nm. maximum thickness, which belied their extreme plasticity. Elementary bodies formed as buds on the surface of mature cells and some began to grow while still attached to the parent cell (Pl. 1, fig. 3). The filamentous forms ramified over areas in excess of 6 μ m. square (Pl. 1, fig. 4). The filaments had an average diameter of 75 nm. and occasionally showed evidence of subdivision into small spheres, believed to be elementary bodies. The elementary bodies measured less than 180 nm. in diameter, the smallest observed being 58 nm. Mycoplasmas which had been exposed to only isotonic conditions and fixed before mounting in negative contrast medium did not differ morphologically from the unfixed specimens. Whereas filaments only occurred intermittently under normal broth culture conditions, exposure to mildly adverse conditions, e.g. 4 to 5° for 16 h. or pH 5 at 37°, resulted exclusively in filament formation.

The large pleomorphic cells with elementary bodies were observed mainly in the stationary phase. Cultures in decline, i.e. older than 5 days, contained increasing numbers of filamentous forms and also particles which were evidently dead mycoplasmas (Pl. 2, fig. 5). These had a shrunken, distorted appearance, an average diameter of 600 nm, and comprised the total population in the 9th and 10th day cultures.

The life cycle of Negroni agent

Immediately after the final filtration with 220 nm. Millipore filter, the filtrate was centrifuged at 30,000 rev./min. in the Spinco rotor 50 for 30 min., and the resultant pellet was resuspended in a 2% (w/v) aqueous solution of phosphotungstate for electron microscopy. Numerous elementary bodies were seen but there were no mature organisms. No significant changes were observed up to 34 h. after incubation, but at 39 and 44 h. a few mature mycoplasmas were present, and when the filtrate was examined after 53 h. of incubation many young, large mature cells were recognized. At approximately 72 h. these mature cells became more pleomorphic. The examination of whole organisms suggested that the life cycle of the Negroni mycoplasma was as depicted in Fig. 1.

Identification of Negroni agent by immunoelectron microscopy

Positive reactions were obtained with mycoplasmas only when they were reacted with their own specific antisera (Pl. 2, fig. 6, 7); pre-immune sera (Pl. 2, fig. 8) as well as all controls were negative. No cross-reactions occurred between any strains or with the antisera of closely related strains, e.g. between *Mycoplasma pulmonis* (M1) and *M. pulmonis* (Negroni) (Pl. 2, fig. 9).

Fig. 1. A suggested scheme of the life cycle of the Negroni strain. (a) Large pleomorphic cell; (b) filamentous form; (c) elementary body (E.B.); (d) dead cell.

DISCUSSION

Observations by immunoelectron microscopy

Fallon *et al.* (1965) first suggested some relationship of Negroni agent to *Mycoplasma pulmonis*. A close relationship of Negroni agent and strain 880 to *M. pulmonis* was confirmed by other laboratories by means of growth inhibition and complement-fixation tests (Butler & Leach, 1966), nucleic acid homology method (Somerson *et al.* 1967) and accessory factor-dependent neutralization test in HEp2 tissue cultures (Sabin, 1967). Nevertheless, a study carried out by Fallon & Jackson (1967) with several strains of *M. pulmonis*, namely Negroni agent, 880, Kon, Cheng, M1 and M2, showed that these strains were, although related, quite distinct from each other. Their conclusions were based mainly on gel diffusion, metabolic inhibition, complement-fixation and growth inhibition tests. Our present observations using immunoelectron microscopy also give support to the opinion of Fallon & Jackson (1967), i.e. no serological cross-reactions were found between any strains of *M. pulmonis*. In contrast to the numerous reports on the use of the immunoferritin technique to identify viruses and bacteria, none has appeared so far concerning the identification of mycoplasma (Andres, Hsu & Seegal, 1967).

The life cycle

Workers on the life cycle of mycoplasmas fall into two principal groups (reviewed by Hayflick & Chanock, 1965). Members of one group support the theory put forward by Freundt (1960) which describes filament growth, the formation of an arborizing

mycelium and its subsequent fragmentation into elementary bodies, whereas members of the other group, e.g. Klieneberger-Nobel (1962), believe that filaments are preparative artefacts resulting from the extreme plasticity of the cell membrane. Though members of the second group accept that filaments grow under certain environmental conditions, they do not consider filament formation to be a major stage in mycoplasma growth. This view has been supported by Reuss working on the Negroni strain (Reuss, 1967). In the present study, the mycoplasma was prepared under conditions isotonic with the culture medium, and the cells were fixed before subjecting them to the rigours of negative contrast, thus preventing gross osmotic distortion. Filaments still occurred under these conditions, and therefore cannot be interpreted as preparative artefacts. This view also agrees well with the work of Hummeler, Tomassini & Hayflick (1965) on the Negroni strain. In cultures at low temperature or low pH, filamentous forms became predominant. It seems, therefore, very likely that filamentary reproduction of elementary bodies is secondary to the budding phenomenon, and the latter should be interpreted as the more normal method of multiplication in *Mycoplasma pulmonis*. According to Dutta *et al.* (1965), *M. gallisepticum* strain s6 appeared to complete its life cycle within 30 to 36 h. The cycle included development of nucleus-like structures in the mother cells, budding from the cell surface, and enlargement of daughter cells, possibly equivalent to elementary bodies, in the final stage. A discrepancy in the cycle time observed in the present report (39 to 53 h.) may be a species difference.

REFERENCES

- ANDRES, G. A., HSU, K. C. & SEEGAL, B. C. (1967). Immunoferritin technique for the identification of antigens by electron microscopy. In *Handbook of Experimental Immunology*, p. 527. Edited by D. M. Weir. Oxford and Edinburgh: Blackwell Scientific Publications.
- BEALE, A. J., CHRISTOPHINIS, G. J. & NEGRONI, G. (1968). Mycoplasma in leukaemia. *British Medical Journal* **i**, 580-581.
- BUTLER, M. & LEACH, R. H. (1966). Mycoplasma associated with tissue cultures, leukaemia and human tumours. *Proceedings of the Royal Society of Medicine* **59**, 1116-1117.
- DE HARVEN, E. & FRIEND, C. (1964). Structure of virus particles partially purified from the blood of leukaemic mice. *Virology* **23**, 119-124.
- DOUGLAS, S. D., GOTTLIEB, A. J., STRAUSS, A. J. L. & SPICER, S. S. (1966). Selectivity of ferritin-protein conjugates for sites on skeletal muscle. *Experimental and Molecular Pathology* **5** (suppl. 3), 5-20.
- DUTTA, S. K., DIERKS, R. E. & POMEROY, B. S. (1965). Electron microscopic studies of the morphology and the stages of development of *Mycoplasma gallisepticum*. *Avian Diseases* **9**, 241-251.
- FALLON, R. J., GRIST, N. R., INMAN, D. R., LEMCKE, R. M., NEGRONI, G. & WOODS, D. A. (1965). Further studies of agents isolated from tissue cultures inoculated with leukaemic bone marrow. *British Medical Journal* **ii**, 388-391.
- FALLON, R. J. & JACKSON, D. K. (1967). The relationship between a rodent mycoplasma, *Mycoplasma pulmonis*, and certain mycoplasmas isolated from tissue cultures inoculated with material from patients with leukaemia. *Laboratory Animals* **1**, 55-64.
- FALLON, R. J. & JACKSON, D. K. (1968). Relation between mycoplasmas and leukaemia and related diseases. *British Medical Journal* **iv**, 225-228.
- FREUNDT, E. A. (1960). Morphology and classification of the PPLO. *Annals of the New York Academy of Sciences* **79**, 312-325.
- FURNESS, G. (1968). Analysis of the growth cycle of *Mycoplasma orale* by synchronized division and by ultraviolet irradiation. *Journal of Infectious Diseases* **118**, 436-442.
- GRACE, J. T., HOROSZEWICZ, J. S., STIM, T. B., MIRAND, E. A. & JAMES, C. (1965). Mycoplasma (PPLO) and human leukaemia and lymphoma. *Cancer* **18**, 1369-1376.

T. TANAKA AND D. A. WOODS

(Facing p. 286)

- GRIST, N. R. & FALLON, R. J. (1964). Isolation of viruses from leukaemic patients. *British Medical Journal* ii, 1263.
- HAYFLICK, L. & CHANOCK, R. M. (1965). Mycoplasma species of man. *Bacteriological Reviews* 29, 185-221.
- HUMMELER, K., TOMASSINI, N. & HAYFLICK, L. (1965). Ultrastructure of a mycoplasma (Negroni) isolated from human leukemia. *Journal of Bacteriology* 90, 517-523.
- INMAN, D. R., WOODS, D. A. & NEGRONI, G. (1964). Electron microscopy of virus particles in cell cultures inoculated with passage fluid from human leukaemic bone marrow. *British Medical Journal* i, 929-931.
- KLIENEBERGER-NOBEL, E. (1962). *Pleuropneumonia-like Organisms (PPLO) Mycoplasmataceae*. New York: Academic Press.
- MILLONIG, G. (1962). Further observations on a phosphate buffer for osmium solutions in fixation. In *Electron Microscopy*, vol. 2, p. 8. Edited by S. E. Breese. New York: Academic Press.
- MOROWITZ, H. J. & MANIOFF, J. (1966). Analysis of the life cycle of *Mycoplasma gallisepticum*. *Journal of Bacteriology* 91, 1638-1644.
- NEGRONI, G. (1964). Isolation of viruses from leukaemic patients. *British Medical Journal* i, 927-929.
- REUSS, K. (1967). Influence of fixation on gross morphology of Mycoplasma. *Journal of Bacteriology* 93, 490-492.
- REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* 17, 208-212.
- SABIN, A. B. (1967). Nature and source of Mycoplasma in various tissue cultures. *Annals of the New York Academy of Sciences* 143, 628-640.
- SINGER, S. J. & SCHICK, A. F. (1961). The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin. *Journal of Biophysical and Biochemical Cytology* 9, 519-537.
- SOMERSON, N. L., REICH, P. R., CHANOCK, R. M. & WEISSMAN, S. M. (1967). Genetic differentiation by nucleic acid homology. III. Relationship among Mycoplasma, L-forms and bacteria. *Annals of the New York Academy of Sciences* 143, 9-20.

EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Extracellular mycoplasma cells in an infected foetal kidney cell culture. $\times 55,000$.
- Fig. 2. An aggregate of extracellular virus-like mycoplasma particles in an infected foetal kidney cell culture. $\times 150,000$.
- Fig. 3. Elementary bodies budding from the large pleomorphic mature cells. $\times 50,000$.
- Fig. 4. Branching filaments growing from a mycoplasma cell. $\times 40,000$.

PLATE 2

- Fig. 5. Mycoplasma particles from a declining 9 day culture. $\times 35,000$.
- Fig. 6. An immunoelectron micrograph of *Mycoplasma pulmonis* (Negroni) reacted with rabbit anti-Negroni agent serum (L). $\times 130,000$.
- Fig. 7. An immunoelectron micrograph of *Mycoplasma pulmonis* (strain 880) reacted with rabbit anti-880 serum (G). $\times 130,000$.
- Fig. 8. An immunoelectron micrograph of *Mycoplasma pulmonis* (Negroni) reacted with rabbit pre-immune serum (L). $\times 120,000$.
- Fig. 9. An immunoelectron micrograph of *Mycoplasma pulmonis* (M1) reacted with rabbit anti-Negroni agent serum (L). $\times 120,000$.